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The overexpression of members of the ErbB family of receptor tyrosine kinases has been implicated in different types of cancer, including breast cancer. Heregulin is the physiological ligand for ErbB3 and ErbB4, and upon ligand binding, these receptors can then dimerize with and activate ErbB2. In breast cancers where ErbB2 is overexpressed, the heregulin signal is constitutively active, even in the absence of ligand. Thus, understanding how heregulin signals are transduced in the cell and what cellular functions these signals are impacting upon should help us to identify molecular points for therapeutic intervention . Our work has identified the nuclear cap-binding complex (CBC) as a novel target for heregulin-stimulated signal transduction processes. The CBC plays a fundamental role in the regulation of gene expression at the level of RNA processing by influencing RNA splicing, export and 3'-end processing events. Thus, it would appear that heregulin can impact upon these RNA metabolic events and we have demonstrated that the heregulin treatment of cells can induce RNA splicing. We now know that the heregulin signal is transmitted to the CBC via the Cdc42, FRAP and S6 kinase proteins. The heregulin treatment of cells also leads to a phosphorylation of the 80 kDa subunit of the CBC, suggesting a possible mechanism for CBC activation. We have shown that the S6 kinase can phosphorylate the CBC in vitro, and based on this information, we have constructed CBP80 mutants to identify the in vivo phosphorylation site, and determine whether this phosphorylation is essential for CBC activation.

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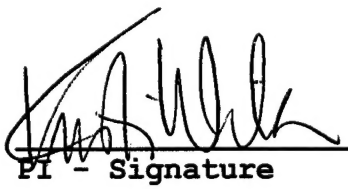

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TABLE OF CONTENTS

INTRODUCTION

Members of the epidermal growth factor (EGF) receptor subfamily of receptor typrosine kinases, including Neu/ErbB2, ErbB3 and ErbB4, have been associated with the development of different cancers, including breast cancer (1-6). Heregulin is the known ligand for this class of receptors (7-9), interacting directly with ErbB3 and ErbB4, and inducing a dimerization with and activation of ErbB2. The overexpression of Neu/Erb2, which presumably causes the constitutive activation of signaling pathways downstream from this receptor, has been correlated with a poor prognosis for women diagnosed with breast cancer (2, 4). Despite the underlying relevance of heregulin and ErbB2 to human breast cancer, relatively little is known regarding the specific heregulin/ErbB2-stimulated signaling pathways that lead to the nucleus and mediate cell growth regulation. This prompted us to look for heregulin-sensitive molecules which might be responsible for mediating heregulin effects on cell growth. The original proposal focused on an 18 kDa nuclear protein which we had identified based on its ability to respond to the heregulin treatment of cells with an increased ability to incorporate GTP as assayed by a photo-catalyzed crosslinking procedure. This molecule was sensitive not only to heregulin, but also to other growth factors, cell stress agents, and in association with the G1/S phase of the cell cycle. It was believed, therefore that the molecular identification and characterization of this molecule would be important both in furthering our understanding of how heregulin effects cellular function on a nuclear level, and also as an endpoint readout to aid in the dissection of heregulin-stimulated signaling processes. We have since identified this molecule as the 20 kDa subunit of the nuclear RNA cap-binding complex (CBC). The CBC, which is comprised of a stable heterodimer between the 20 kDa subunit and an 80 kDa subunit (CBP20 and CBP80, respectively), binds to the ^{7}methylguanosine cap structure on RNAs transcribed by RNA polymerase II (10). This binding facilitates gene expression at fundamental levels of RNA processing: it enhances the splicing of capped mRNAs (10-13), it facilitates in the nuclear export of U snRNAs (14), and it is involved in an early step of the polyadenylation of mRNAs (15). The fact that the recognition of capped RNAs by the CBC can be regulated by growth factors including heregulin, suggests that heregulin can mediate gene expression by influencing RNA metabolic processes in which the CBC is involved. To further strengthen this view, we have demonstrated that heregulin addition to quiescent cells can stimulate the splicing of capped RNAs (16). Over the last year, we have focused our efforts on understanding the chain of signaling events which culminate in an activation of the CBC. We now know that the CBC can become activated downstream of a signaling pathway utilizing Cdc42, FRAP, and S6 kinase. Additionally, we have demonstrated that CBP80 becomes phosphorylated *in vivo* in response to growth factors, and this activation can be blocked by a small molecule inhibitor, rapamycin, which is known to block FRAP activation. The parallels between CBC activation and CBP80 phosphorylation suggest that this phosphorylation event may be critical in activating the CBC. *In vitro* studies show that the S6 kinase is capable of phosphorylating CBP80, and we have mapped this phosphorylation to the nuclear localization sequence in the N-terminus of CBP80. We have therefore constructed as series of mutations in CBP80 and are currently in the process of determining whether the N-terminus is the *in vivo* site of phosphorylation of CBP80, and if so, whether this phosphorylation is essential for CBC activation in response to heregulin.

BODY

Experimental procedures

This section will contain some of the more routinely used experimental procedures which are relevant to the results described below.

1. Cell culture conditions and cell lysis

Mammalian cells were maintained as described in Appendix 1. As indicated, cells are switched to serum free medium for 40-48 hrs, and/or treated with NGF (100 ng/ml, Gibco/BRL). Transfection experiments were performed according to the manufacturer's directions using the Lipofectamine Plus reagent (Gibco BRL). Mammalian expression constructs used include: pcDNA3 HA-L61 Cdc42, pcDNA3 HA-wild type Cdc42, pcDNA3 HA-L28 Cdc42, pDCR HA-V12 Ras, pCMV HA-myristoylated 110 kDa PI3 kinase catalytic subunit, and pJ3H myc-pp70^{S6K}. Concentrated stocks of rapamycin and wortmannin (Sigma) were prepared in ethanol. Prior to addition, the appropriate volume of inhibitor was subaliquoted, the ethanol was evaporated and the drug was resuspended in a small volume of DMEM. Cells were then treated with the inhibitor for 30 minutes at concentrations indicated in the text. Cell fractionation and lysate preparation were as described in appendix 1.

To metabolically label PC12 or HeLa cells, cells were washed once with phosphate free DMEM, and then incubated with phosphate free DMEM containing 1.5 mCi/ml ^{32}P orthophosphoric acid (150 mCi/ml, NEN) for 3 hours followed by NGF and/or rapamycin treatment as indicated in the text. Lysis buffers for this procedure included the addition of 50 mM β-glycerolphosphate and 50 mM NaF to the previously described cytosolic and nuclear lysis buffers.

2. Photoaffinity Labeling Assay

CBC cap-binding activity was assayed by measuring the incorporation of [α^{32}P]GTP into CBP20 by UV crosslinking as established in appendix 1.

3. Immunoprecipitation and Western Immunoblotting

Immunoprecipitation and Western immunoblotting were performed as described in appendix 1. Antibodies used include an antiserum raised against CBP80 (10), and monoclonal antibodies recognizing hemaglutinin- and myc-tags.

4. Generation of CBP80 mutants

CBP80 N-terminal deletion mutants and point mutants were generated by PCR using wildtype CBP80 cDNA as template. For the deletion mutant (ΔN13 CBP80), a 5' primer was designed to amplify the CBP80 gene without the bases coding for the first 13 amino acids. To generate point mutants (T21A, S22A CBP80; T21D, S22D CBP80; K17A, R18A CBP80), 5' and 3' primers were designed to amplify a small portion (~100 bases) of the 5'-end of CBP80, introducing the indicated mutations. This product was then used as the 5' primer for a subsequent round of amplification of the entire gene. All PCR reactions were carried out for 40 cycles (30 sec at 94°C, 30 sec at 55°C, 2 min at 72°C) using the pfu enzyme (Clontech). The CBP80 mutant PCR products were then subcloned into the mammalian expression vectors pKH3 and pcDNA3.

Results and Discussion

The goal of the original proposal was to identify novel heregulin-stimulated cellular activities, with the premise that the identity of such molecules could shed light on how heregulin regulates normal and abnormal cellular function. Additionally such molecules, if

they function at some position downstream of receptor activation, could be used to dissect out heregulin-activated signal transduction pathways. The original proposal was centered around a nuclear GTP-binding activity which we had discovered, termed p18, which was found to be activated in response to the heregulin treatment of serum-arrested cells. Furthermore, this activity was also found to be stimulated in response to other growth factors, such as nerve growth factor (NGF), by cellular stress agents such as UV irradiation, and in association with the G1/S phase of the cell cycle. The focus of the progress report submitted last year was the identity of this nuclear, heregulin-activated protein as the 20 kDa subunit of the nuclear cap-binding complex (CBC), CBP20 (for cap-binding protein, 20 kDa). CBP20, together with its binding partner CBP80 (80 kDa, cap-binding protein) comprise a complex with binds to the ^{7}methylguanosine ($m^{7}G$) cap structure on eucaryotic RNAs transcribed by RNA polymerase II (10). This subset of RNAs includes messenger RNAs and U snRNAs. Previous work by others had demonstrated that the CBC plays a fundamental role in gene expression by greatly enhancing the splicing of capped mRNAs (10-13), by facilitating the nuclear export of U snRNAs (14), and by stimulating the 3'-end polyadenylation process (15). Our finding, then, that the CBC could be stimulated to bind capped RNAs in response to heregulin and other growth factors, suggests that such agents can influence cellular gene expression at the level of RNA processing. Indeed, we demonstrated that the addition of heregulin to serum-arrested HeLa cells could induce the splicing of capped RNAs (16). These results directly addressed Task 2 in the original Statement of Work (Purification and Molecular Cloning of p18) and can be reviewed in our publication resulting from this work found in appendix 1 (16).

Also in this publication and in our last report, we had begun to address issues raised in Task 1, involving the signal transduction elements necessary in the activation of p18 (from hereon referred to as CBP20 or the CBC). We had demonstrated that the low molecular weight GTP-binding proteins, Cdc42 and Ras, were both capable of activating the CBC when introduced into cells in a constitutively active state. This activation could be attenuated by treating cells with a small molecule, rapamycin (17), whose cellular target is known to be the FKBP12-rapamycin associated protein (FRAP) (18-22). Thus, FRAP can be placed downstream of Cdc42 and Ras in a signaling pathway leading to the CBC. This result is of interest because FRAP has been previously implicated in translational control by its ability to regulate 4E-BP1 (a negative regulator of translational initiation) (23-25) and pp70 S6 kinase (26, 27), suggesting a possible intersection between translational control and RNA processing. In the closure of our last report we stated our observation that S6 kinase was capable of phosphorylating the CBC *in vitro,* and suggested that S6 kinase might function downstream of Cdc42 in cells to phosphorylate the CBC, and that such a phosphorylation could provide a mechanism by which the CBC could become activated to bind capped RNAs in response to growth factors such as heregulin. For the remainder of this report, we will focus on our current work aimed at refining the role of a Cdc42 signaling pathway in CBC activation, and the role of phosphorylation as a mechanism of activation for the CBC.

As stated above, we have seen that the small molecule rapamycin can block an activation of the CBC by either constitutively active Cdc42 or Ras. We next wanted to compare the efficacy of another small molecule inhibitor, wortmannin (which inhibits the PI3 kinase), with rapamycin for its ability to block CBC activation and thus address the question of whether the PI3 kinase might also be involved in signaling to the CBC. Therefore, HeLa cells were transiently transfected with a myc-tagged L28 Cdc42 or a HA-tagged V12 Ras construct and then starved for 48 hours. Thirty minutes prior to harvesting, rapamycin and wortmannin were added to the cells. As expected, rapamycin caused a decrease in the activation of the CBC by either L28 Cdc42 or V12 Ras as indicated by a decrease in [α^{32}P]GTP crosslinking to CBP20 (see Figure 1). Wortmannin, on the other hand, yielded no effect. This suggests that either PI3 kinase does not participate in

signaling pathways leading to an activation of the CBC, or if it does, it functions upstream of Cdc42 and Ras.

The S6 kinase is a cellular protein whose activation is blocked by the rapamycin treatment of cells placing its activation downstream of FRAP (26). Similarly, PI3 kinase is important in yielding an active S6 kinase as the addition of wortmannin to cells can also block the activation of S6 kinase (28, 29), and the phosphatidylinositol-3 dependent kinase-1 (PDK1) has been shown to phosphorylate and activate S6 kinase (30, 31). To determine whether the rapamycin sensitive activation of the CBC is a result of a FRAP-S6 kinase pathway or an alternative FRAP pathway, it was important to further explore the possibility that PI3 kinase could activate the CBC. To this end, Cos cells were transiently transfected with a constitutively active PI3 kinase construct, an HA-tagged myristoylated form of the 110 kDa catalytic subunit of PI3 kinase. Additionally, we transfected a myc-tagged, wild type S6 kinase, or contransfected the wild type S6 kinase and myristoylated 110 kDa subunit of PI3 kinase together. The transfected cells were then starved for 48 hours, and the cytosolic lysates were assayed for the presence of the transfected proteins by Western blotting, and the nuclear lysates were assayed for CBC activation by the incorporation of [α^{32}P]GTP into CBP20 (Figure 2A and B). Wild type S6 kinase did not lead to a high activation of the CBC. However, constitutively active PI3 kinase caused a significant increase in CBC activity compared to the activation observed in response to wild type S6 kinase. This activation was further enhanced by cotransfection of S6 kinase with constitutively active PI3 kinase even though less of the 110 kDa PI3 kinase subunit was expressed under cotransfection conditions, suggesting that the constitutively active PI3 kinase is activating the wild type S6 kinase to yield an active CBC. Together these data outline a signaling pathway to the CBC which utilizes FRAP and S6 kinase downstream of either Cdc42 or Ras.

A further question regards the mechanism of activation of the CBC in response to growth factors. The cytosolic cap-binding protein, eIF-4E, becomes rapidly phosphorylated in response to growth factors (32), and this phosphorylation correlates with eIF-4E activation *in vivo* and an increase of cap-binding by eIF-4E *in vitro* (33). To examine whether phosphorylation might also play a role in the activation of the CBC, we asked whether either CBP20, CBP80, or both were phosphorylated in response to growth factors *in vivo*. PC12 cells were labeled with ^{32}P for 4 hours and then stimulated with NGF for 15 minutes. The CBC was then immunoprecipitated from the cytosolic and nuclear lysates using a specific CBP80 antiserum. Preimmune serum was used as a nonspecific control. The 80 kDa subunit of the CBC becomes phosphorlyated in response to NGF treatment of PC12 cells (Figure 3). Phosphorylation of CBP20 was not observed. PC12 cells were used in these experiments because in their undifferentiated state, these cells are quite small, and thus contain a proportionally high nuclear content. A high nuclear content is necessary to obtain enough material to immunoprecipitate the CBC from, while keeping the cell culture volume reasonable so that we can perform these experiments which require large amounts of radioactive phosphorous. However, similar results have also been obtained using heregulin to stimulate HeLa cells. Thus, a growth factor-dependent phosphorylation of CBP80 correlates with a growth factor-dependent activation of the CBC.

As we have demonstrated that the activation of the CBC can be inhibited by the addition of rapamycin to cells, we were interested in determining whether the *in vivo* phosphorylation of CBP80 could similarly be blocked by rapamycin. PC12 cells were again labeled with ^{32}P. Prior to a 15 minute NGF treatment, cells were treated with rapamycin for 30 minutes. CBP80 was then immunoprecipitated from the cytosolic and nuclear lysates to determine its phosphorylation state. As was the case in the previous experiment, CBP80 became phosphorlyated in response to NGF treatment. Pretreatment with rapamycin blocked the NGF-dependent phosphorylation (Figure 4). It appears, therefore, that the phosphorylation of CBP80 utilizes a FRAP signaling pathway as does the activation of the CBC. The growth factor and rapamycin-sensitive phosphorylation of

CBP80 correlates well with the functional activation of the CBC. Phosphorylation could directly induce a conformational change by the CBC which would result in a CBC functional to bind capped RNAs, although we think this is unlikely as the *in vitro* phosphorylation of the recombinant CBC by S6 kinase does not influence cap binding. Alternatively, a phosphorylation could recruit or inhibit binding partners to the CBC which, in turn, would be responsible for regulating the RNA binding. Determination of the residue(s) on CBP80 which become phosphorylated *in vivo* will be necessary for ascertaining the functional significance of the phosphorylation of CBP80 as it pertains to cap-binding by the CBC.

As mentioned earlier, the rapamycin-sensitive kinase, S6 kinase, can phosphorylate the CBC *in vitro*. CBP80 contains two possible S6 kinase phosphorylation sites residing in its extreme N-terminus. Interestingly, these sites correspond to the nuclear localization sequence of CBP80. Using a CBP80 mutant where a lysine and an arginine within the second S6 kinase phosphorylation site have been mutated to alanine (K17A, R18A), we identified the site *in vitro* where S6 kinase phosphorylates CBP80 (Figure 5). We have thus constructed a series of CBP80 mutants (see "Experimental Procedures") to test the hypothesis that this *in vitro* S6 kinase phosphorylation site is also the *in vivo*, growth factor-responsive phosphorylation site, and if it is, that the phosphorylation of this site is necessary for the growth factor induced activation of the CBC. We are currently testing this hypothesis by transfecting these mutants into cells and (1) examining the ability of these mutants to become phosphorylated *in vivo* as compared to wildtype CBP80 and (2) examining the effect of these mutants on the ability of the CBC to respond to growth factors by binding capped RNA. If we determine that this is the site of phosphorylation *in vivo*, we additionally want to examine the possibility that S6 kinase is the *in vivo* kinase for CBP80 by transfecting activated S6 kinase constructs into cells and looking for the ability of CBP80 to become phosphorylated in the absence of growth factors.

A phosphorylation within the NLS of CBP80 would most likely effect the interaction between the CBC and the importins. The importin heterodimer (comprised of an α and β subnunit) is necessary for the nuclear import of proteins containing classical, basic nuclear localization sequences (34). Importin α recognizes the NLS and importin β, bound to importin α, is responsible for docking at the nuclear pore. Subsequently, the transport complex is translocated into the nuclear lumen in a step that requires Ran•GTP (35). Within the nucleus, the importins dissociate from their cargo and are recycled back to the cytoplasm (36). An NLS phosphorylation of CBP80 would presumably inhibit importin binding. We find phosphorylated CBP80 in the nucleus of cells, but not it the cytoplasm even though the CBC is thought to shuttle between the two compartments. Previous work has shown that the interaction between the importins and the CBC can modulate CBC capped RNA-binding. Specifically, the CBC can bind to capped RNA when associated with importin α, but not when bound to the α/β heterodimer (37). A nuclear phosphorylation of CBP80 within its NLS would prevent the binding of α/β importin and thus ensure that CBC-bound RNA would not be released within the nucleus. Once in the cytosol, release of the RNA and dephosphorylation would have to occur before the CBC could be reimported into the nucleus for another cycle. Such a model would causally link growth factor signaling, CBC capped RNA-binding, and CBC nucleocytoplasmic transport. Thus far, we find that the CBP80 mutant, ΔN13 T21D, S22D CBP80, which should mimic a CBP80 which is constitutively phosphorylated within the second S6 kinase phosphorylation site, cannot localize to the nucleus, whereas a mutant which does not contain the aspardic acid substitutions (ΔN13 CBP80) demonstrates proper nuclear localization. Therefore, it would appear that such a phosphorylation would indeed disrupt an interaction between the importins and the CBC and support the hypothesis mentioned above.

It is possible, however, that these NLS mutants will respond normally to growth factors with regard to activation and/or phosphorylation. In the event that the current mutants are still able to become phosphorylated in response to heregulin *in vivo*, we will

continue to construct other CBP80 mutants (based on other putative phosphorylation sites within the CBP80 sequence) in a further attempt to identifiy the *in vivo* phosphorylation site. Once this site is identified, a determination of the importance of this phosphorylation to CBC activation will be made. If these mutants fail to become phosphorylated *in vivo*, but are still activated to bind capped RNAs in response to growth factors, we would conclude that this phosphorylation is not the mechanism by which a growth factor signal is conveyed to the CBC in order to activate it. In this event, we will pursue the possibility that the CBC is interacting with a heregulin-sensitive regulatory protein(s) which is responsible for its activation, and seek to identify this protein using an affinity chromatography approach. A GST-CBC column will be constructed, and used to bind CBC regulatory proteins out of heregulin-stimulated cell lysates. If we can identify CBC-binding proteins (as assessed by silver staining), we will scale up this procedure and attempt to purify these proteins in sufficient quantities for microsequencing analysis. Such proteins would then be assessed biochemically for their ability to activate the CBC.

Recommendations in relation to the Statement of Work

As has been addressed previously, Task 2 of the original proposal has been completed with the identification of p18 as CBP20 followed by the molecular cloning of both subunits, CBP20 and CBP80, of the CBC. We have developed expression systems for these proteins in mammalian, bacterial and insect cell systems. We are currently focusing our efforts on Task 1 and Task 3. Task 1 addresses the involvement of Cdc42 signaling pathways in the activation of p18 (now known to be the CBC). We had shown previously that Cdc42 is indeed capable of activating the CBC, and proposed last year that S6 kinase might be the Cdc42 effector through which the signal is being conveyed. We have now confirmed that S6 kinase, in a rapamycin-dependent manner, is functioning downstream of Cdc42 in this capacity. It will be of further interest to examine different Cdc42 effector mutants for their ability to support CBC activation. This is of interest in many regards. Such information will shed light upon the manner in which Cdc42 is conveying information to the CBC via S6 kinase. Does the communication directly involve an interaction between Cdc42 and S6 kinase as has been previously reported (38)? Given that rapamycin can block the activation of the CBC (suggesting FRAP is downstream of Cdc42), it is possible that Cdc42 might couple directly with FRAP. Such an interaction would highlight a novel signaling interaction. Alternatively, other known Cdc42 effectors such as PAK or IQGAP might be playing a bridging role between Cdc42, FRAP and S6 kinase. Effector mutational analysis should aid in a deliniation between these possibilities. Cdc42 has been shown previously in our lab to cause cellular transformation when introduced into cells in a constitutively active state (39). Additionally, we have shown that a Cdc42 mutant which lacks an insert region particular to Rho family members, is not able to cause cellular transformation (40). The Cdc42 target necessary which is not able to bind this mutant has not yet been identified, and we are excited by the possibility that this region might be critical to signaling to the CBC. If this appears to be the case, then the CBC would therefore be implicated as a cellular target of Cdc42 necessary for its growth regulation capabilities.

Task 3 addresses the biochemical regulation of the CBC. It mainly centers around examining interactions between the CBC and RCC1, and trying to identify a guanine nucleotide exchange factor for the CBC. As stated in last year's report, as originally conceived, these are no longer viable avenues to pursue. Namely, we cannot detect an interaction between the CBC and RCC1, and CBP80 turned out to be the 80 kDa activator which we had originally proposed. Nonetheless, we have continued in the spirit of this Task by attempting to identify the mechanism by which the CBC is activated in response to growth factors. We have shown that the CBC can become phosphorylated in response to growth factors, and believe that this phosphorylation, by regulating and interaction between the CBC and the importins, is a possible mechanism of activation. As described above,

our studies are currently directed to examine this possibility, and we have taken into consideration contingent strategies to further investigate how the CBC becomes activated by growth factors if it turns out not to be through a phosphorylation event.

Finally we, in collaboration with Dr. Jon Clardy's laboratory here at Cornell University, are attempting to identify the three-dimensional structure of the CBC by X-ray crystallography. Though this is well beyond the scope of the original proposal, we are excited by the possibility that in the future, detailed structural information might aid in the development of precise modulators of CBC activity which might ultimately be able to be tested as candidate therapeutic reagents.

KEY RESEARCH ACCOMPLISHMENTS

* The CBC, a protein complex involved in gene regulation at the level of RNA processing, has been identified as a novel endpoint for heregulin-stimulated signal transduction pathways.

* Heregulin can stimulate the splicing machinery in quiescent cells.

* The signal transdution pathway to the CBC utilizes the GTP-binding protein Cdc42 upstream of FRAP and S6 kinase.

* The CBP80 subunit of the CBC is subject to a rapamycin-sensitive phosphorylation in response to growth factors, suggesting a possible mechanism for CBC activation.

* The S6 kinase phoshorylates the CBC *in vitro*.

REPORTABLE OUTCOMES

* A publication on this work appeared in the Journal of Biological Chemistry (See appendix I)

* The primary investigator received her Ph.D. in Biochemistry, Cellular and Molecular Biology from Cornell University in May, 1999.

* The primary investigator applied and received offers for postdoctoral positions in the following laboratories:
  Dr. Gideon Dreyfuss, University of Pennsylvania School of Medicine
  Dr. John Lis, Cornell University
  Dr. Rey Chen, Cornell University
  Dr. Richard Cerione, Cornell University
  and is currently continuing her work as a postdoctoral associate in the lab of Dr. Richard Cerione.

CONCLUSIONS

The overexpression of the ErbB2 and ErbB3 receptor tyrosine kinases is an important factor in many breast cancers. Indeed, up to 40% of all breast cancers show an increase in ErbB2 levels (1-6). The physiological ligand for these receptors has been shown to be the glycoprotein growth factor, heregulin (7-9). An interaction between heregulin and ErbB3 induces a heterodimerization between ErbB3 and ErbB2 which results in the transphosphorylation and activation of the ErbB2 receptor (41). The phosphorylation of ErbB2 serves to initiate signaling cascades which in turn can impact upon normal cell function, growth and division. When ErbB2 is overexpressed, it becomes constitutively activated thereby bypassing regulation by heregulin. It is presumed that this persistent heregulin signal in the absence of ligand contributes to the occurrence of breast cancer. Therefore, it has been the objective of this study to gain a better understanding of how heregulin signaling occurs in a normal cell and what cellular functions heregulin can effect. The rationale for such an objective is that the more that is understood about the molecules involved in heregulin signaling in a normal cell, the greater the probability that cellular targets for therapeutic intervention can be identified.

In the original proposal, we described a novel nuclear target for heregulin signaling which responded to the growth factor treatment of cells with an increased ability to be labeled with GTP. We postulated that this activity might function in the nucleus to transduce a heregulin signal to impact upon nuclear function. Subsequently, we identified this activity as the 20 kDa subunit of the nuclear cap-binding complex (CBC), and demonstrated that the CBC is stimulated to bind to capped RNAs in response to heregulin (16). The CBC, by recognizing the ^{7}methylguanosine cap structure on mRNAs and U snRNAs, has been shown previously to be an important factor involved in multiple levels of RNA metabolism including RNA splicing, export, and 3'-end processing (10, 14, 15). This identification suggests that heregulin can impact upon cell growth by modulating gene expression at the level of RNA processing via the CBC and indeed, we have demonstrated that heregulin can stimulate the splicing of capped RNAs. This observation opens up the possibility that in a malignant situation where the heregulin signal is constitutive, the active CBC endpoint can effect gene expression by amplifying the rate of RNA processing or by otherwise altering the RNA transcript profile, and thus contributes to unregulated cell growth and division.

The identification of the CBC as a target for heregulin signaling not only gives us information about how heregulin might effect cell growth, but it also serves as a convenient endpoint which we can use to identify components of the heregulin signaling pathway to the CBC. To this end, we have recently started to piece together such a pathway which includes the low molecular weight GTP-binding protein Cdc42, FRAP, and S6 kinase. Activated forms of Cdc42 themselves can be transforming (39), which suggests that the overactivation of Cdc42 in ErbB2 cancers might be an important component in the malignant transformation.

We have also been working toward a molecular understanding of the mechanism by which heregulin and other growth factors activate the CBC. We have demonstrated that the CBP80 subunit of the CBC undergoes a rapamycin-sensitive phosphorylation in response to the growth factor treatment of cells. Interestingly, we have also found that the rapamycin-sensitive kinase, S6 kinase can phosphorylate the CBC *in vitro*. Because S6 kinase also leads to the activation of the CBC *in vivo*, this leads to the possibility that the S6 kinase is phosphorylating the CBC *in vivo*, and that this phosphorylation is necessary for the growth factor regulated activation of the CBC. We have mapped the *in vitro* phosphorylation site to the N-terminal nuclear localization sequence of CBP80 and are in the process of determining whether this is the *in vivo* phosphorylation site as well. Our ongoing efforts will continue to focus on the elucidation of the signaling pathways which lead to this novel heregulin target, the CBC, as well as on the molecular mechanism of

activation of the CBC. We believe that such efforts will increase our knowledge of the molecular participants in heregulin signaling and thus increase the likelihood that one or more of these identified participants may be a suitable target for therapeutic intervention.

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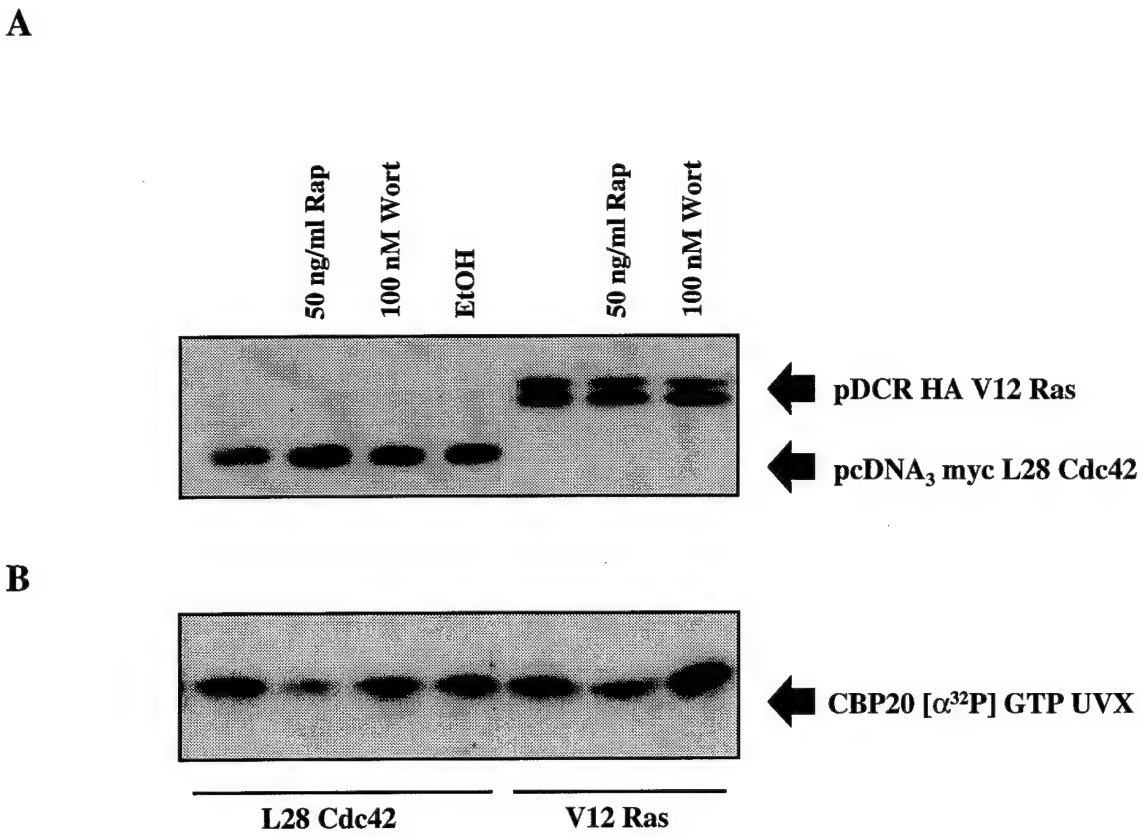

Figure 1. The activation of the CBC by Cdc42 and Ras can be blocked by the small molecule inhibitor, rapamycin. NIH 3T3 cells were transiently transfected with constitutively active Cdc42 F28L (*L28 Cdc42, lanes 2-4*) or GTPase defective Ras G12V (*V12 Ras, lanes 5-7*) and serum starved for 48 hours prior to a 30 minute treatment with rapamycin (*Rap, lanes 2* and *6*) or wortmannin (*Wort, lanes 3* and *7*). The cytoplasmic lysates were examined for the expression of the Cdc42 or Ras proteins using antibodies directed against the myc- or HA- tags of the recombinant proteins (A). The nuclear lysates were assayed for CBC activation by photoaffinity labeling CBP20 with [α^{32}P]GTP (B).

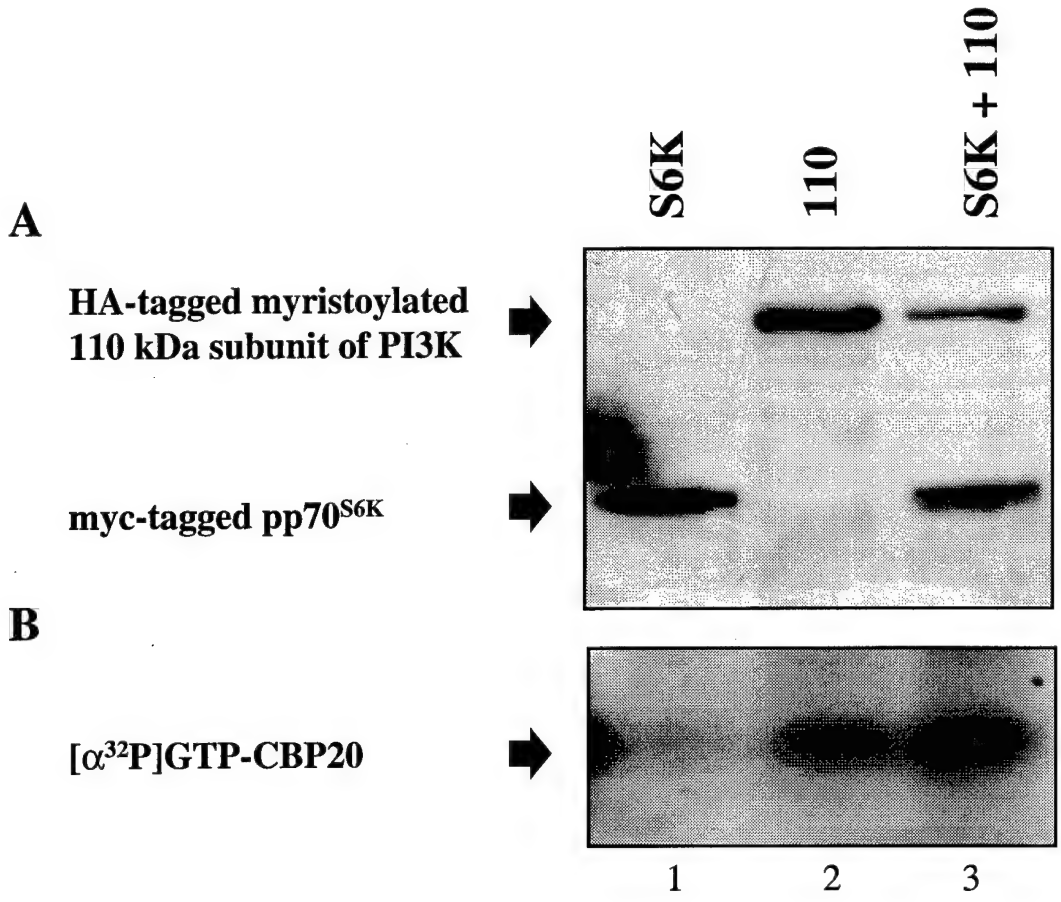

Figure 2. Constitutively active P13 kinase and S6 kinase stimulate the CBC.
COS-7 cells were transiently transfected with wild type S6 kinase (*S6K, lanes 1* and *3*) and/or a constitutively active, myristoylated 110 kDa catalytic subunit of PI3 kinase (*110, lanes 2* and *3*) and serum starved for 48 hours. The cytosolic lysates were assayed for the presence of the S6 kinase and the myristoylated 110 kDa subunit of PI3 kinase using antibodies directed against the myc- and HA-tags on the recombinant proteins (A). CBC activity in the nucleus was assayed by [α^{32}P]GTP crosslinking to CBP20 (B).

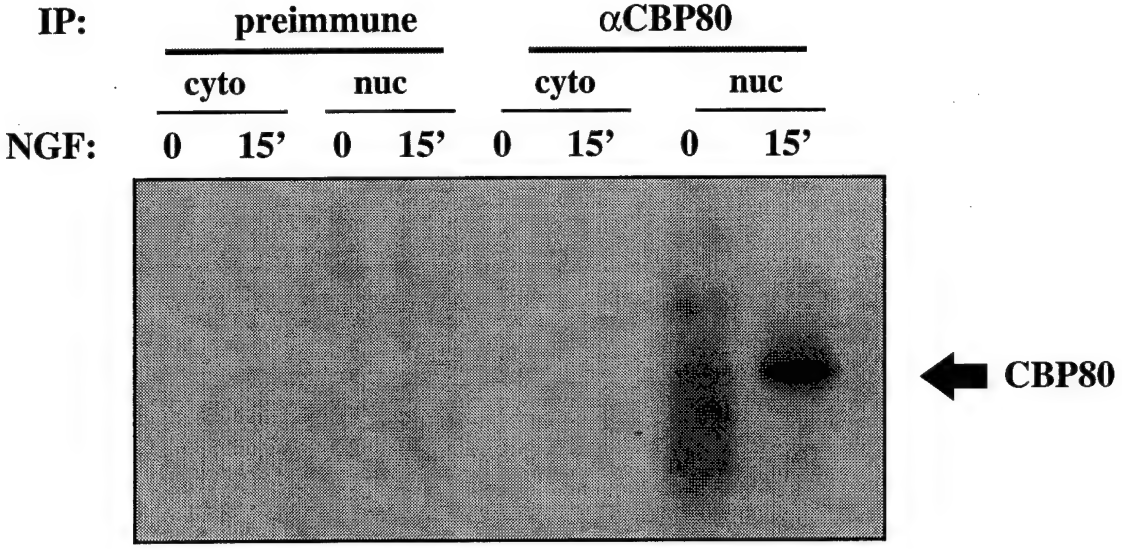

Figure 3. The 80 kDa subunit of the CBC, CBP80, is phosphorylated *in vivo* in response to the NGF treatment of PC12 cells.

PC12 cells were metabolically labeled with ^{32}P orthophosphoric acid for 3 hours and then stimulated with NGF (100 ng/ml) for 15 minutes. The cells were harvested and fractionated into cytosolic and nuclear lysates, and these lysates were then immunoprecipitated using either a specific CBP80 antiserum, or a nonspecific preimmune rabbit antiserum. Immunoprecipitated proteins were separated by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography.

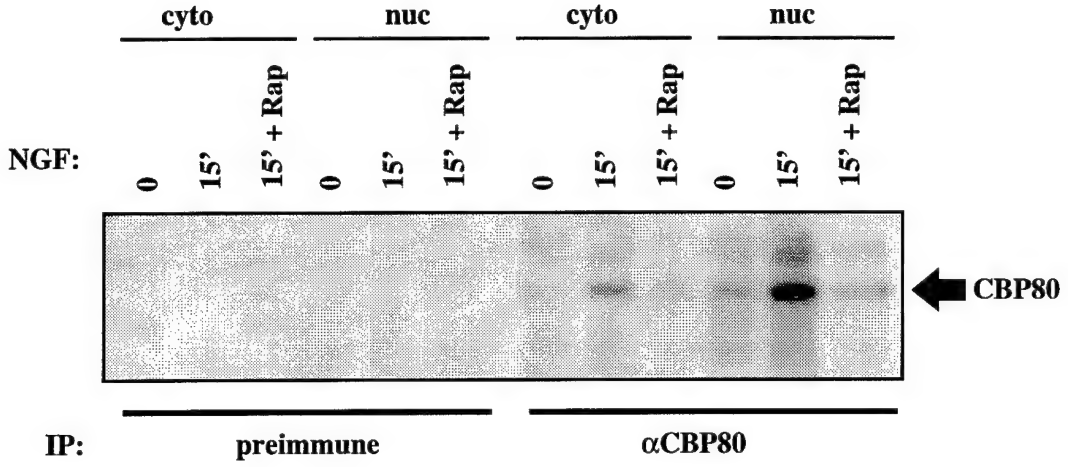

Figure 4. The NGF-stimulated phosphorylation of CBP80 is inhibited by rapamycin.

PC12 cells were metabolically labeled for 3 hours with ^{32}P orthophosphoric acid. Thirty minutes prior to NGF treatment (100 ng/ml, 15 minutes), 50 ng/ml rapamycin (*Rap*) was added as indicated. The cells were harvested and fractionated, and the cytosolic and nuclear lysates were immunoprecipitated using either a specific CBP80 antiserum, or a nonspecific, rabbit preimmune antiserum. Immunoprecipitated proteins were separated by SDS-PAGE, and phosphorylated proteins were detected by autoradiography.

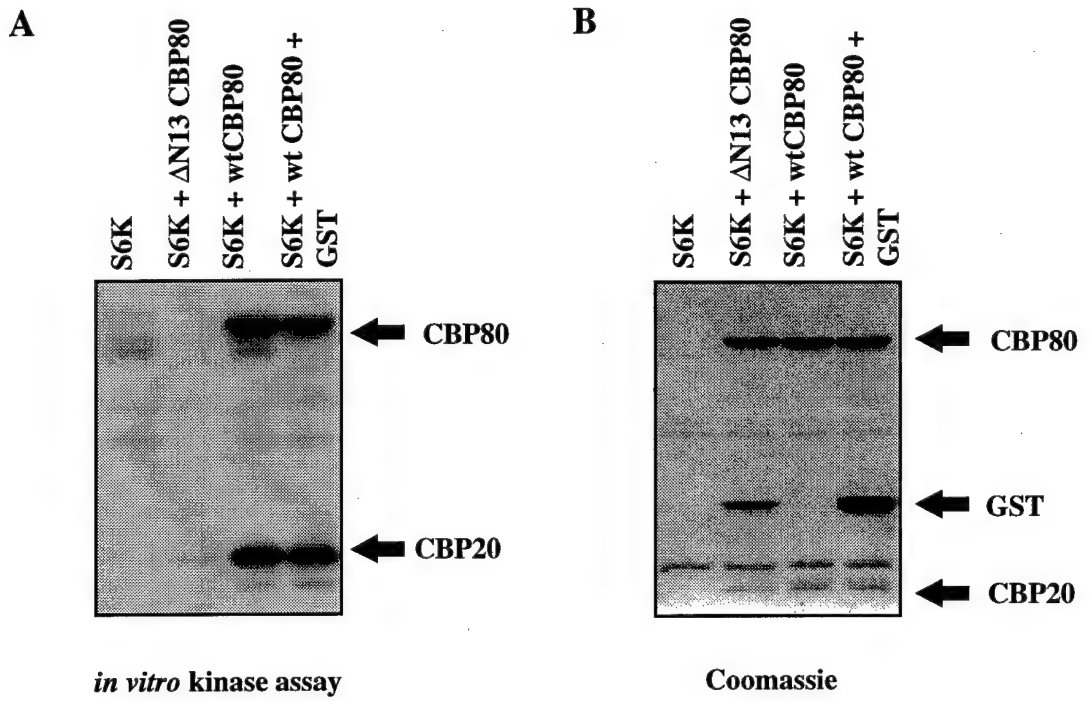

Figure 5. pp70^{S6K} cannot phosphorylate CBC proteins in which the second putative S6 kinase phosphorylation site of CBP80 is disrupted.

CBC proteins (wild type (*wt*) or an NLS mutant of CBP80 (*NLS2*)) were assayed for their ability to become phosphorylated by pp70^{S6K} *in vitro*. The CBC proteins were then detected by Coomassie staining (B) or by autoradiography (A).

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The Nuclear Cap-binding Complex Is a Novel Target of Growth Factor Receptor-coupled Signal Transduction*

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In an attempt to further understand how nuclear events (such as gene expression, nuclear import/export, and cell cycle checkpoint control) might be subject to regulation by extracellular stimuli, we sought to identify nuclear activities under growth factor control. Using a sensitive photoaffinity labeling assay that measured [α-^{32}P]GTP incorporation into nuclear proteins, we identified the 20-kDa subunit of the nuclear cap-binding complex (CBC) as a protein whose binding activity is greatly enhanced by the extracellular stimulation of serum-arrested cells. The CBC represents a 20- and 80-kDa heterodimer (the subunits independently referred to as CBP20 and CBP80, respectively) that binds the 7-methylguanosine cap on RNAs transcribed by RNA polymerase II. This binding facilitates precursor messenger RNA splicing and export. We have demonstrated that the [α-^{32}P]GTP incorporation into CBP20 was correlated with an increased ability of the CBC to bind capped RNA and have used the [α-^{32}P]GTP photoaffinity assay to characterize the activation of the CBC in response to growth factors. We show that the CBC is activated by heregulin in HeLa cells and by nerve growth factor in PC12 cells as well as during the G_1/S phase of the cell cycle and when cells are stressed with UV irradiation. Additionally, we show that cap-dependent splicing of precursor mRNA, a functional outcome of CBC activation, can be catalyzed by growth factor addition to serum-arrested cells. Taken together, these data identify the CBC as a nuclear target for growth factor-coupled signal transduction and suggest novel mechanisms by which growth factors can influence gene expression and cell growth.

Growth factor binding to cell-surface receptors can initiate signals that are propagated through the cell by a cascade of protein-protein interactions, ultimately to impact upon specific cellular functions and regulate cell growth. The activities of signaling molecules must be tightly regulated to maintain the integrity of cellular communication, as loss of regulation in these processes can give rise to defects in cell growth and metabolism that may lead to human disease. Given the importance of signaling processes in cell growth, a great deal of effort has gone into the elucidation of proteins participating in signaling pathways that start at the level of receptor activation and culminate in the stimulation of a nuclear activity. Multiple cascades have now been identified that result in the activation of different nuclear mitogen-activated protein kinases, including the extracellular receptor-activated kinases and the stress-responsive c-Jun N-terminal kinase/stress-activated protein kinase and p38 (1, 2). Extracellular receptor-activated kinase activation is the outcome of mitogen-stimulated Ras signaling, whereas c-Jun N-terminal kinase/stress-activated protein kinase and p38 activities are often stimulated by pathways involving the Cdc42 and Rac GTP-binding proteins (2–6). Although these different signaling pathways were originally thought to be independently regulated, later work showed that cross-talk between the individual mitogen-activated protein kinase pathways exists. A common functional outcome of the activation of these signaling pathways is a translocation of the activated mitogen-activated protein kinase to the nucleus and subsequent activation of specific transcription factors and gene expression (2–6).

How other nuclear functions might be influenced in response to extracellular stimulation is less clear. However, it is attractive to envision how critical nuclear activities such as RNA metabolism and export, nuclear protein import, and cell cycle control might be subject to regulation as downstream targets of extracellular stimuli. With this in mind, we set out to identify novel nuclear activities that were growth factor-responsive. Using a photoaffinity labeling approach, we identified the nuclear cap-binding complex (CBC)[1] as such an activity based on the enhanced ability of its ~20-kDa subunit (CBP20) to undergo a photocatalyzed incorporation of [α-^{32}P]GTP in response to extracellular stimulation. The CBP20 protein and its 80-kDa binding partner, CBP80, constitute a functional CBC (7–10). This nuclear complex binds cotranscriptionally to the monomethylated guanosine cap structure (m^7G) of RNA polymerase II-transcribed RNAs (7, 11, 12) and has been reported to play a role in diverse aspects of RNA metabolism: it increases the splicing efficiency of cap proximal introns (7, 13–15), positively affects the efficiency of 3′-end processing (16), and is required for the efficient transport of U snRNAs (9). We demonstrate that the incorporation of [α-^{32}P]GTP by CBP20 reflects the activation of the CBC and is correlated with its ability to bind capped RNA. A variety of growth factors and other cellular stimuli can activate the CBC under conditions that can give

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[1] The abbreviations used are: CBC, cap-binding complex; NGF, nerve growth factor; EGF, epidermal growth factor; TBS, Tris-buffered saline; DTT, dithiothreitol; AMP-PNP, adenosine 5′-(β,γ-iminotriphosphate); GMP-PNP, guanosine 5′-(β,γ-iminotriphosphate); PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; HA, hemagglutinin; eIF, eukaryotic initiation factor; snRNA, small nucleotide RNA.

rise to a stimulation of the splicing of precursor mRNAs in an *in vitro* assay system. The implications of CBP20 functioning as a novel end point in signal transduction highlight the importance of RNA metabolism in regulated cell growth.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions—Rat pheochromocytoma (PC12) cells were maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 10% horse serum, and antibiotic/antimycotic solution (Sigma). All other cell types, including HeLa, BHK21, and COS-7 cells, were maintained in Dulbecco's modified Eagle's medium with the addition of 10% fetal bovine serum and antibiotic/antimycotic solution. Prior to growth factor treatment, cells were switched to serum-free medium for 40 h. Growth factors (NGF (Life Technologies, Inc.), heregulin β1 (residues 177–244; a generous gift from Dr. Mark Sliwkowski, Genentech), and EGF (Calbiochem) or 25% fetal bovine serum) were then added to the serum-free medium in the concentrations and for the times indicated under "Results" at 37 °C. Following treatment, the growth factor-containing medium was removed, and the cells were washed twice with Tris-buffered saline (TBS; 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, and 1.0 mM EDTA) and then lysed (see below). Cell cycle blocks were performed in HeLa cells. A G_0 block was achieved by switching to serum-free medium for 22–24 h. For G_1/S phase arrest, 2.5 mM thymidine was added to the growth medium for 22–24 h. 80 ng/ml nocodazole was added to the growth medium for 22–24 h to achieve arrest in M phase. After treatment, cells were collected, washed twice with TBS, and lysed. To challenge cells with UV irradiation, the medium was removed from serum-starved cells, and the cells were then exposed to UV light for 2 min. Following exposure, cells were replenished with serum-free medium and allowed to recover at 37 °C for the times indicated below.

Cell Fractionation and Nuclear Lysis—Tissue culture cells were washed twice on the plate with TBS and then lysed in a buffer containing Hanks' solution (20 mM Hepes, pH 7.4, 5 mM KCl, 137 mM NaCl, 4 mM $NaHCO_3$, 5.5 mM D-glucose, and 10 μM EDTA), 0.3% (v/v) Nonidet P-40, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin. The lysate was then centrifuged at 800 rpm for 15 min at 4 °C. The supernatant was microcentrifuged for 10 min at 4 °C, and then the resulting supernatant was saved as the cytoplasmic fraction. The nuclear pellet was washed twice with an equal volume of Hanks' solution with 0.2% (v/v) Triton X-100 and centrifuged at 800 rpm for 15 min at 4 °C. The resulting pellet was treated as the purified nuclear fraction. The nuclei were then lysed in a buffer containing 50 mM Tris, pH 7.4, 1% (v/v) Triton X-100, 400 mM KCl, 1 mM sodium orthovanadate, 1 mM DTT, and protease inhibitors as described above. The samples were incubated on ice for 30 min and microcentrifuged for 10 min at 4 °C, and the supernatant was used as the whole nuclear fraction. For nuclear fractionation, nuclei were isolated from tissue culture cells, and nuclear membranes and nuclear soluble fractions were then prepared as described by Davis and Blobel (17) with some modification. The whole nuclear fraction was resuspended in 50 mM Tris-HCl, pH 7.4, 10% (w/v) sucrose, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM $MgCl_2$, and protease inhibitors. DNase-I (5 mg/ml) and RNase A (1 mg/ml) were added, and the nuclei were then incubated for 15 min at 37 °C. Following the incubation with DNase-I, the nuclei were underlaid with 30% sucrose and then subjected to centrifugation in a swinging bucket rotor for 10 min at 20,000 × *g* to generate a soluble nuclear fraction and a nuclear membrane fraction.

Photoaffinity Labeling with [α-^{32}P]GTP—Photoaffinity labeling of cellular proteins with [α-^{32}P]GTP was performed as described previously (18). In brief, the UV cross-linking reaction was carried out in a buffer containing 50 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM DTT, 20% (v/v) glycerol, 100 mM NaCl, and 500 μM AMP-PNP. Samples (20 μl) prepared from the cell fractionation procedures, described above, were incubated for 10 min at room temperature with an equal volume of cross-linking buffer containing [α-^{32}P]GTP (2–3 μCi/sample, 3000 Ci/mmol; NEN Life Science Products) in a 96-well, non-tissue culture-treated plate. The samples were then placed in an ice bath and irradiated with UV light (254 nm) for 15 min. After irradiation, samples were mixed with 5× Laemmli buffer and boiled. SDS-PAGE was performed using 15% acrylamide gels. The gels were then typically silver-stained and dried, and autoradiography was performed (typically overnight) using Kodak X-Omat XAR-5 film at −80 °C. To perform competition experiments, competing nucleotides (m^7GpppG and GpppG (New England Biolabs Inc.) and m^7GTP and GTP (Sigma)) were added to the sample prior to the addition of the [α-^{32}P]GTP-containing cross-linking buffer. This buffer did not contain AMP-PNP. The samples were then subjected to UV cross-linking as described above.

Purification of an 18-kDa Protein from Bovine Retinal Tissue That Incorporates [α-^{32}P]GTP—Bovine retinas were obtained frozen from J. A. & W. L. Lawson Co. (Lincoln, NE). The retinas (typically 200/batch) were thawed in a buffer containing 50 mM Tris, pH 8.0, 25 mM KCl, 5 mM $MgCl_2$, and protease inhibitors as described for cell lysate preparations and then homogenized with a motor-driven Dounce homogenizer. The homogenate was centrifuged at 2500 rpm in a swinging bucket rotor to yield a crude nuclear pellet. The nuclei were purified from this crude preparation using the method described by Blobel and Potter (19), and the soluble nuclear contents were then extracted as described above. The 18-kDa activity was precipitated using 40–75% ammonium sulfate, resuspended in 3–5 ml of Buffer A (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20 mM KCl), and loaded onto a fast protein liquid chromatography Superdex-200 Highload 16/60 column as described above. The purification of this activity was monitored by both silver staining and UV cross-linking to [α-^{32}P]GTP. The fractions eluted from the Superdex-200 column were assayed for [α-^{32}P]GTP incorporation into the 18-kDa protein, and six peak fractions (eluting with molecular masses of ~100–150 kDa) were pooled in a final volume of 12 ml and loaded directly onto a fast protein liquid chromatography ion-exchange Mono Q 5/5 column (Amersham Pharmacia Biotech) equilibrated in Buffer A minus KCl. Bound proteins were eluted from the Mono Q 5/5 column with a 28-ml linear gradient of 100–500 mM NaCl. [α-^{32}P]GTP-incorporating activity eluted from the Mono Q 5/5 column with ~300 mM NaCl in a volume of 5 ml. Peak activity as assayed by [α-^{32}P]GTP incorporation was eluted from the Mono Q column and applied directly to a Bio-Gel HPHT hydroxylapatite column (Bio-Rad) equilibrated in 10 mM potassium phosphate, pH 6.8, 2.5 mM $MgCl_2$, 0.01 mM $CaCl_2$, and 1 mM DTT. Bound proteins were then eluted, first by stepping the potassium phosphate to 100 mM and then by a 20-ml linear gradient of 100–300 mM potassium phosphate. Peak activity as assayed by the light-catalyzed incorporation of [α-^{32}P]GTP was found to elute with ~250 mM phosphate.

Cloning and Expression of Recombinant CBP20—CBP20 was cloned by polymerase chain reaction from HeLa cell cDNA (a generous gift from Dr. Wannian Yang, Cornell University). 5′- and 3′-primers were designed using the published sequence for *Homo sapiens* CBP20 (GenBank™ accession P52298), and the *CBP20* gene was then amplified from the HeLa cell cDNA using 40 polymerase chain reaction cycles (1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C). The 470-base product was inserted into a cloning vector (pCR2.1) using a TA cloning kit (Invitrogen) and then subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and into the *Escherichia coli* expression vector pGEX-2TK.

E. coli cells transformed with the pGEX-2TK-CBP20 vector were grown in a 1-liter culture, and expression of glutathione *S*-transferase (GST)-CBP20 protein was induced for 3 h using isopropyl-β-D-thiogalactopyranoside. Following induction, the cells were pelleted by centrifugation (5000 rpm for 10 min in a JA-10 rotor). The harvested cells were resuspended in 15 ml of 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 1 mM DTT, and protease inhibitors (as described above) and then lysed using 15 mg of lysozyme, followed by the addition of 200 mM $MgCl_2$ and 1 mg of DNase-I. Following centrifugation (100,00 × *g* for 30 min at 4 °C), the supernatant was incubated with glutathione-agarose beads for 1 h at 4 °C to bind the GST-CBP20 protein. Glutathione-agarose-bound CBP20 was washed with 50 mM Tris-HCl, pH 8.0, 0.5% (v/v) Triton X-100, 200 mM KCl, and 1 mM DTT and then stored in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 μM GTP, and protease inhibitors. GST-CBP20 was eluted from the glutathione-agarose beads using 10 mM glutathione, pH 8.0, and the GST moiety was cleaved from CBP20 by the addition of 500 units of thrombin for 30 min at room temperature.

Using the LipofectAMINE protocol (Life Technologies, Inc.), a hemagglutinin-tagged form of CBP20 (HA-CBP20) was transiently transfected into BHK21 cells according to the manufacturer's directions. Following a 5-h incubation with serum-free medium containing the lipid-DNA complex, the medium was removed and replaced with medium containing 10% fetal bovine serum. Cells were allowed to grow in the presence of serum for ~20 h and were then switched to serum-free medium for 40 h prior to stimulation with serum.

Immunoprecipitation and Western Immunoblotting—A polyclonal antibody generated against recombinant CBP80 (αCBP80) was prepared as described previously (7). Cytosolic and nuclear lysates were prepared as described above. Prior to immunoprecipitation, the cytosolic lysate was adjusted to 100 mM NaCl, and the nuclear lysate was

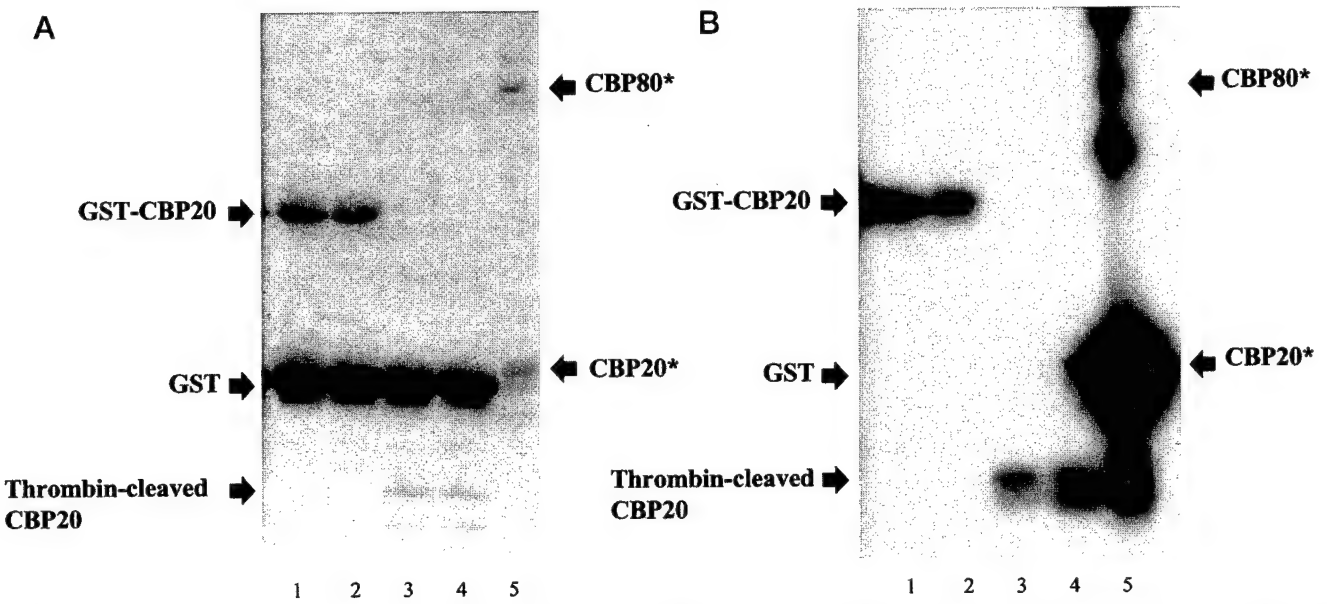


FIG. 1. **Recombinant *E. coli*-expressed CBP20 incorporates [α-^{32}P]GTP in a photoaffinity labeling assay.** CBP20 was expressed and purified from *E. coli* as a GST fusion protein. GST-CBP20 (*lanes 1* and *2*), free CBP20 (the GST was cleaved with thrombin) (*lanes 3* and *4*), and *E. coli*-expressed CBC (complex proteins designated as *CBP80** and *CBP20**) (*lane 5*) were then assayed for their ability to incorporate [α-^{32}P]GTP. Following UV cross-linking, proteins were separated by 15% SDS-PAGE and visualized by Coomassie Blue staining (*A*) and autoradiography (*B*).

diluted 3-fold with 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 1 mM sodium orthovanadate. The lysates were then allowed to incubate at 4 °C for 1 h, with or without the addition of 5 μl of 12CA5 monoclonal antibody or αCBP80 polyclonal antibody. Following the first incubation, 40 μl of protein A-Sepharose beads were added to each sample, and the samples were incubated for another hour at 4 °C. The samples were then centrifuged, and the immunoprecipitated pellets were washed four times with 50 mM Tris-HCl, pH 8.0, 133 mM KCl, 0.33% Triton X-100, 1 mM DTT, and 1 mM sodium orthovanadate. The resulting immunoprecipitated pellets were resuspended in 20 μl of UV cross-linking buffer and were incubated with [α-^{32}P]GTP and UV cross-linked as described above.

For Western blot analysis, proteins were transferred to polyvinylidene difluoride membranes following SDS-PAGE. The polyvinylidene difluoride membranes were blocked with 2.5% (w/v) bovine serum albumin in TBS plus 0.1% Tween 20 for 1 h at room temperature. After blocking, the membranes were incubated with either 12CA5 or αCBP80 antibody for 1 h at room temperature, washed with several changes of TBS and 0.1% Tween 20, and incubated for 30 min at room temperature with sheep anti-rabbit or sheep anti-mouse horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech) as appropriate. Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RNA Binding Assays—UV cross-linking was done essentially as described by Rozen and Sonenberg (20), except that the RNA probe was transcribed from *Bam*HI-cleaved pBluescript II KS with T3 RNA polymerase (Promega).

Pre-mRNA Splicing Reactions—Splicing extracts were prepared from HeLa cells (serum-starved for 40 h prior to stimulation with 100 nM heregulin for 24 h) as described by Lee and Green (21). pBSAd1 precursor linearized by *Sau*3AI was transcribed using T3 RNA polymerase in the presence of m^7GpppG dinucleotide cap. Splicing reactions were then carried out as described by Izaurralde *et al.* (7). In brief, 60 μg of splicing extract were preincubated for 15 min at 30 °C with 1 mM $MgCl_2$. 5 mM creatine phosphate, 1.5 mM ATP, 2.5×10^4 cpm of labeled precursor mRNA, and an additional 1 mM $MgCl_2$ were then added in a final volume of 20 μl, and the reactions were incubated for 2 h at 30 °C. Splice products were visualized by separation on a 10% denaturing polyacrylamide gel, followed by autoradiography.

RESULTS

The overall goal of these studies was to identify nuclear activities that could represent novel downstream targets in receptor-coupled signaling pathways. One of the assays we used to identify such activities was the photocatalyzed incorporation of [α-^{32}P]GTP into nuclear proteins. The rationale for this approach was that it would provide a very sensitive assay for identifying guanine nucleotide-binding activities in the nucleus, in a manner analogous to the use of phosphorylation assays to identify growth factor-sensitive phosphosubstrates. Using this assay, we identified an 18-kDa protein that strongly incorporated [α-^{32}P]GTP in serum-treated but not serum-starved cells (see below). We found this activity to be exclusively nuclear and present in every cell line we examined, including HeLa, PC12, COS-7, and BHK21 cells, as well as in various mammary epithelial cells. A similar activity was also observed in the yeast *Saccharomyces cerevisiae*.

A purification scheme was developed using bovine retinal nuclei, which were a particularly rich source of this 18-kDa nuclear activity. A series of three chromatography steps resolved the activity, as assayed by [α-^{32}P]GTP incorporation, from the majority of contaminating low molecular mass proteins (see "Experimental Procedures"). These steps also resolved an 80-kDa protein (designated p80), detected by silver staining, which co-purified with the 18-kDa activity. This putative protein complex was reminiscent of the nuclear CBC, as the CBC comprises an 18-kDa nuclear protein, CBP20 (for cap-binding protein 20), stably complexed with an 80-kDa protein, designated CBP80. The formation of the CBP20-CBP80 heterodimer enables the CBC to bind a guanine derivative, the 7-methylguanosine cap structure (m^7GpppN), on RNAs transcribed by RNA polymerase II (7–10). The similarities between the 18-kDa nuclear activity and CBP20 (both in complex formation and substrate binding) led us to investigate whether the CBC was a nuclear target for extracellular signals.

First, we assayed directly the ability of recombinant *E. coli*-expressed CBP20 to incorporate [α-^{32}P]GTP. Fig. 1*A* shows GST-CBP20, thrombin-cleaved CBP20, and the complexed CBC proteins (His-tagged CBP20 (9) and CBP80 (7)) as visualized by staining with Coomassie Blue. Fig. 1*B* shows that the recombinant CBP20 proteins were all capable of incorporating [α-^{32}P]GTP in a photoaffinity labeling assay. This activity was greatly enhanced by the presence of CBP80 (see *lane 5*), consistent with previous studies that have demonstrated that complex formation between CBP20 and CBP80 is necessary for

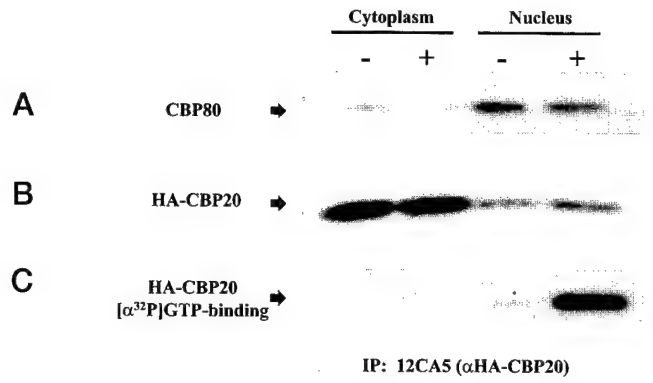
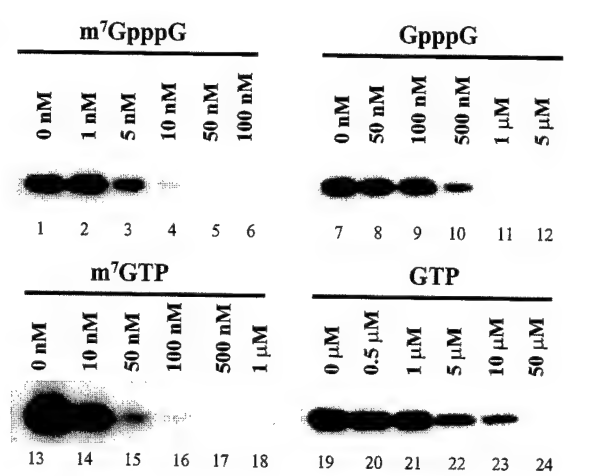


FIG. 2. **Serum-dependent incorporation of [α-^{32}P]GTP into recombinant HA-CBP20 expressed in BHK21 cells.** Human CBP20 was cloned by polymerase chain reaction from HeLa cell cDNA and then subcloned into the mammalian expression vector pcDNA3 to express a HA-tagged form of the protein. BHK21 cells were transiently transfected with HA-CBP20 (using 8 μg of DNA/100-mm plate of BHK21 cells). The transfected cells were serum-starved for 40 h (−) and then stimulated with 25% fetal bovine serum (+) for 1.5 h. HA-CBP20 was immunoprecipitated (*IP*) from cytosolic or nuclear lysates using 12CA5 monoclonal antibody. Immunoprecipitates were then assayed for [α-^{32}P]GTP incorporation. Proteins were separated by 15% SDS-PAGE and transferred to Immobilon for Western blot analysis and autoradiography. *A* shows the CBP80 protein co-immunoprecipitating with HA-CBP20 from the nuclear lysates as detected by Western blotting using CBP80 antiserum. *B* is a Western blot using 12CA5 antibody to detect immunoprecipitated HA-CBP20 from cytosolic and nuclear lysates. The [α-^{32}P]GTP incorporation corresponding to immunoprecipitated HA-CBP20 is shown in *C*.



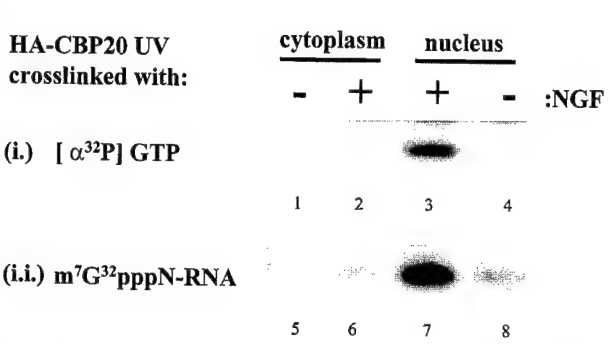


FIG. 3. **Incorporation of [α-^{32}P]GTP into CBP20 reflects an m^{7}GpppN-capped RNA binding event by the CBC that can be growth factor-regulated.** *A*, [α-^{32}P]GTP incorporation into PC12 cell CBP20 is blocked by the addition of RNA cap analogs. Nuclear extracts were prepared from PC12 cells growing asynchronously in culture. 200 μg of lysate were then immunoprecipitated with 5 μl of αCBP80 antiserum. The immunoprecipitates were assayed for [α-^{32}P]GTP incorporation into CBP20 in the presence of m^{7}GpppG (0, 1, 5, 10, 50, and 100 nM (*lanes 1–6*, respectively)), GpppG (0 nM, 50 nM, 100 nM, 500 nM, 1 μM, and 5 μM (*lanes 7–12*, respectively)), m^{7}GTP (0 nM, 10 nM, 50 nM, 100 nM, 500 nM, and 1 μM (*lanes 13–18*, respectively)), and GTP (0, 0.5, 1, 5, 10, and 50 μM (*lanes 19–24*, respectively)). Following cross-linking, proteins were separated by 15% SDS-PAGE; the gel was dried; and autoradiography was performed. *B*, CBP20 binds capped RNA in a growth factor-dependent manner. PC12 cells stably expressing HA-CBP20 were serum-starved (−) and then treated with 100 ng/ml NGF for 1 h (+). After preparation of cytosolic and nuclear lysates, HA-CBP20 was immunoprecipitated from the lysates and assayed in the presence of either [α-^{32}P]GTP (*lanes 1–4*) or m^{7}G^{32}pppN-capped RNA (*lanes 5–8*).

capped RNA binding. The GST control did not show any cross-linking to [α-^{32}P]GTP.

We next examined whether the ability of CBP20 to incorporate [α-^{32}P]GTP could be regulated in response to serum. BHK21 cells were transiently transfected with a HA-tagged CBP20 construct. Following 40 h of serum starvation, the cells were stimulated with 25% fetal bovine serum for 1.5 h, and HA-CBP20 was immunoprecipitated from cytosolic and nuclear lysates prepared from either serum-starved or stimulated cells. The immunoprecipitates were then assayed for the photocatalyzed incorporation of [α-^{32}P]GTP into CBP20. HA-CBP20 was present in both the cytosolic and nuclear fractions (Fig. 2*B*), and CBP80 co-immunoprecipitated with nuclear localized HA-CBP20 equally well under conditions of either serum starvation or stimulation (Fig. 2*A*). The large percentage of HA-CBP20 localized to the cytosol is presumably the result of its overexpression. Nuclear CBP20 demonstrated a marked serum-dependent incorporation of [α-^{32}P]GTP (Fig. 2*C*).

Given that the m^{7}GpppN RNA cap structure is a known substrate for the CBC, the stimulated incorporation of [α-^{32}P]GTP into CBP20 may reflect an enhanced ability of the CBC to bind the cap structure on RNA. To address this issue, we first examined the relative binding affinities of the CBC for different cap analogs by testing their ability to inhibit the incorporation of [α-^{32}P]GTP into CBP20. PC12 cell nuclear lysates were immunoprecipitated with antibodies generated against CBP80 (*i.e.* the binding partner of CBP20) (7), and the immunoprecipitates were then assayed for photocatalyzed incorporation of [α-^{32}P]GTP into CBP20 in the absence and presence of RNA cap analogs or GTP. CBP20 proteins that co-immunoprecipitated with CBP80 could be efficiently labeled with [α-^{32}P]GTP. This activity was strongly inhibited by the addition of low concentrations of cap analogs to the [α-^{32}P]GTP cross-linking assay and yielded the following binding specificity: m^{7}GpppG > m^{7}GTP > GpppG > GTP (Fig. 3*A*). Indeed, the m^{7}GpppG analog competed with [α-^{32}P]GTP for binding to CBP20 ~1000 times more effectively than GTP, suggesting that the CBC most likely binds RNA, rather than GTP, in cells.

We further examined whether the CBC shows a regulated binding to capped RNAs using a PC12 cell line that stably expresses HA-tagged CBP20. Following starvation, these cells were stimulated with NGF. HA-CBP20 was then immunoprecipitated from the cytoplasmic and nuclear lysates and assayed for the incorporation of either [α-^{32}P]GTP (Fig. 3*B*, *upper panel*) or m^{7}G^{32}pppN-capped RNA (*lower panel*). Both substrates were incorporated into nuclear HA-CBP20 strictly in a growth factor-dependent manner. Thus, these findings indicate that the growth factor-stimulated incorporation of [α-^{32}P]GTP into CBP20 accurately reflects the activation of the CBC, such that it is induced to bind m^{7}GpppN-capped RNAs.

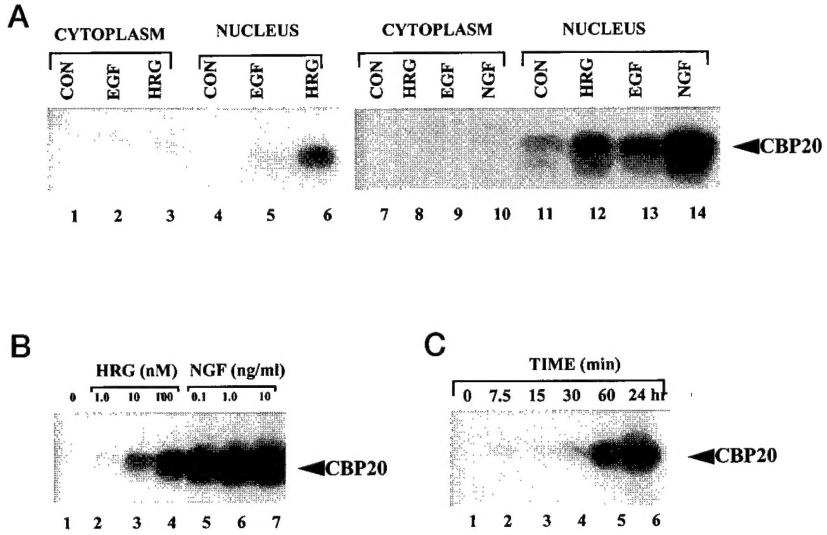


FIG. 4. **Characterization of the growth factor-induced capped RNA-binding activity of the CBC (assayed by CBP20 [α-^{32}P]GTP incorporation).** *A*, HeLa cells (*lanes 1–6*) were serum-starved (control (*CON*); *lanes 1* and *4*) and then treated with 100 ng/ml EGF (*lanes 2* and *5*) or 30 nM heregulin (*HRG*; *lanes 3* and *6*) for 15 min at 37 °C. PC12 cells (*lanes 7–14*) were serum-starved (control; *lanes 7* and *11*) and then treated with 30 nM HRG (*lanes 8* and *12*), 100 ng/ml EGF (*lanes 9* and *13*), or 100 ng/ml NGF (*lanes 10* and *14*). The cells were lysed, separated into cytoplasmic (*lanes 1–3* and *7–10*) and whole nuclear (*lanes 4–6* and *11–14*) fractions, and assayed for [α-^{32}P]GTP incorporation into CBP20 using 45 μg of protein from cell lysates. *B*, a dose-response experiment was performed with the addition of either heregulin (*lanes 2–4*) or NGF (*lanes 5–7*) to serum-starved PC12 cells (control; *lane 1*) for 30 min at 37 °C. For each dose of heregulin or NGF, 50 μg of total nuclear lysate protein were assayed for the incorporation of [α-^{32}P]GTP into CBP20, and then 15% SDS-PAGE was performed. The resulting gel was dried and exposed to x-ray film for 5–15 h. *C*, a time course of 100 ng/ml NGF treatment was performed in serum-starved PC12 cells (control; *lane 1*) with NGF addition for 7.5 min (*lane 2*), 15 min (*lane 3*), 30 min (*lane 4*), 60 min (*lane 5*), or 24 h (*lane 6*). 50 μg of protein from the nuclear lysates were assayed for incorporation of [α-^{32}P]GTP into CBP20, followed by 15% SDS-PAGE and autoradiography overnight.

We took further advantage of the high sensitivity of the [α-^{32}P]GTP incorporation assay to examine the abilities of different growth factors to activate the endogenous CBC. Fig. 4*A* (*left panel*) shows the results obtained when HeLa cells were first serum-starved and then treated with EGF and heregulin (the ligand for the Neu-ErbB2/ErbB3 and Neu-ErbB2/ErbB4 heterodimers (22, 23)). Endogenous CBP20 present in nuclear lysates from HeLa cells was strongly stimulated to incorporate [α-^{32}P]GTP by heregulin as well as, to a lesser extent, by EGF. Similarly, in PC12 cells, endogenous CBP20 present in nuclear lysates was activated by growth factors (Fig. 4*A*, *right panel*). In this case, the incorporation of [α-^{32}P]GTP into CBP20 was most strongly stimulated by NGF (as observed in Fig. 3*B*), followed by heregulin and then EGF. Fig. 4*B* shows that in all cases, the growth factor-stimulated activation of CBP20 was dose-dependent.

In our initial experiments, the incorporation of [α-^{32}P]GTP into CBP20 was assayed after relatively short periods of growth factor treatment (~15 min). Although this was sufficient to detect incorporation of the radiolabeled GTP, more complete time course experiments indicated that near maximal incorporation occurred following treatment with growth factors for 1 h. An example for PC12 cells is shown in Fig. 4*C*. In this experiment, serum-starved PC12 cells were challenged with 100 ng/ml NGF for increasing time periods, up to 24 h. The results show that near maximal incorporation of [α-^{32}P]GTP into CBP20 was observed after ~1 h of growth factor addition and that this level of incorporation was maintained through 24 h. A similar time course was obtained when PC12 cells were treated with heregulin (data not shown).

Nuclear lysates from asynchronously growing cells also contain activated CBP20, suggesting that the growth factor regulation of the CBC activity may be associated with a particular phase of the cell cycle. This is illustrated in Fig. 5*A*. HeLa cells were arrested in G_0 phase by serum starvation, in G_1/S phase by thymidine addition, and in M phase by nocodazole treatment. Cytoplasmic and nuclear fractions were then prepared (or a mitotic pellet was prepared in the case of M phase-arrested cells), and the resulting lysates were assayed for the ability of CBP20 to incorporate radiolabeled GTP. We found that CBP20 did not incorporate [α-^{32}P]GTP in cells arrested in either G_0 or M phase of the cell cycle. However, CBP20 strongly incorporated [α-^{32}P]GTP in HeLa cells arrested in G_1/S phase. Thus, the activation of the CBC appears to be sensitive to cell cycle-dependent as well as growth factor-dependent regulation.

To determine whether the CBC might respond to a broader range of stimuli, we assayed the ability of CBP20 to incorporate radiolabeled GTP under conditions of cellular stress. PC12 cells were first serum-starved and then exposed to UV radiation for 2 min. Following this exposure, the cells were allowed to recover for 30 min or 1 h, and then endogenous CBP20 was assayed for its ability to incorporate [α-^{32}P]GTP. Fig. 5*B* shows that CBP20 was strongly stimulated to incorporate radiolabeled GTP in cells that had been UV-irradiated. We found a similar stress activation of endogenous CBP20 in COS-7 and HEK-293 cells (data not shown).

Stress response pathways have been shown to be mediated by the low molecular mass GTP-binding proteins Cdc42 and Rac and to culminate in transcriptional activation through the stimulation of the nuclear mitogen-activated protein kinases JNK1 and p38/HOG1 (3–5, 24). Thus, we examined whether the transient expression of activated Cdc42 would result in a growth factor-independent activation of the CBC. The results in Fig. 5*C* indicate that this is the case. We found that the transient expression of either a GTPase-defective Cdc42 mutant (Cdc42 Q61L) or a transforming Cdc42 mutant that is capable of undergoing the spontaneous exchange of GTP for GDP (Cdc42F28L) strongly activated CBP20, whereas expression of wild-type Cdc42 showed no activation. We also have found that expression of V12-Ras stimulates the incorporation of [α-^{32}P]GTP into CBP20 as well as activated Rac and RhoA (data not shown), although thus far, Cdc42 appears to be the most effective activator.

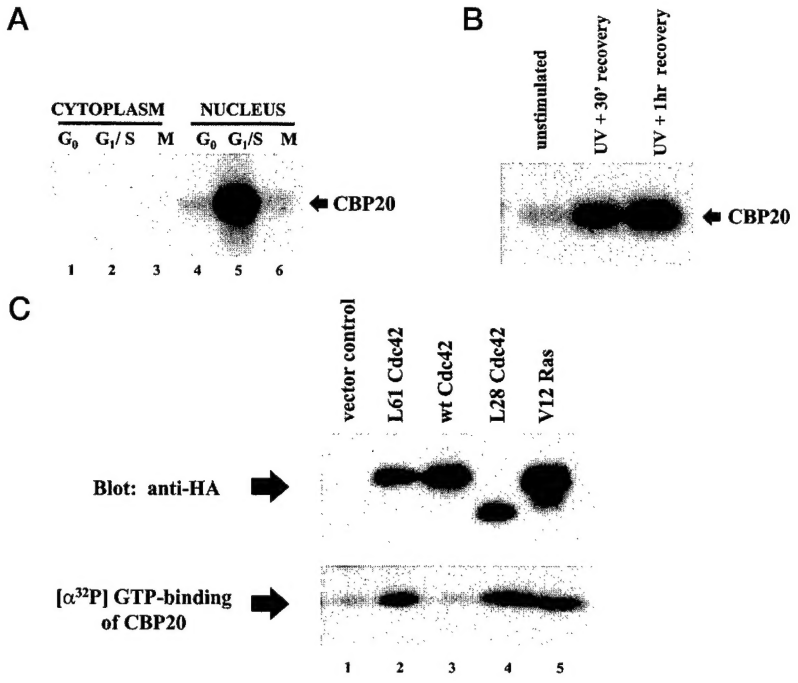


FIG. 5. **The CBC shows a cell cycle- and cell stress-dependent activation and can be activated by the low molecular mass GTP-binding proteins Cdc42 and Ras.** *A*, HeLa cells were arrested in G_0 phase by serum starvation (*lanes 1* and *4*), in G_1/S phase by 2.5 mM thymidine addition (*lanes 2* and *5*), and in M phase with 80 ng/ml nocodazole (*lanes 3* and *6*). The cells were then separated into cytoplasmic (*lanes 1–3*) and whole nuclear (or a mitotic pellet was prepared for M phase arrest) (*lanes 4–6*) fractions, and then for each fraction, 50 μg of protein were assayed for [α-^{32}P]GTP incorporation into CBP20, followed by 15% SDS-PAGE and autoradiography. *B*, PC12 cells were serum-starved and then exposed to UV light for 2 min. Following exposure, cells were replenished with serum-free medium and allowed to recover for 30 min or 1 h. Cells were then harvested; nuclear lysates were prepared; and 50 μg of nuclear lysate protein were assayed for [α-^{32}P]GTP incorporation into CBP20 by 15% SDS-PAGE and autoradiography. *C*, HeLa cells were transiently transfected with GTPase-defective Cdc42 Q61L (*L61 Cdc42*), wild-type (*wt*) Cdc42, constitutively active Cdc42 F28L (*L28 Cdc42*) , or GTPase-defective Ras G12V (*V12 Ras*) for 24 h, followed by serum starvation for 40 h. The cytoplasmic lysates were analyzed for expression of the transfected proteins by Western blotting using an anti-HA antibody directed against the HA tag on the recombinant GTP-binding proteins (*upper panel*). The nuclear lysates were assayed for [α-^{32}P]GTP incorporation into CBP20 (*lower panel*).

Taken together, these data suggest that the ability of the CBC to bind RNA cap structures is a tightly regulated process. Previous work by others has defined a role for CBC binding to capped RNAs in important RNA metabolic processes, including pre-mRNA splicing (7, 13–15), U snRNA export (9), and 3′-end processing (16). The ability of growth factors to stimulate the capped RNA-binding activity of the CBC suggests that those metabolic processes that benefit from the recognition of the RNA cap by the CBC (such as pre-mRNA splicing) will also be subject to extracellular regulation. To test this prediction, splicing extracts were prepared from HeLa cells that were either serum-starved or starved and then stimulated with heregulin for 24 h (*i.e.* conditions that lead to maximal stimulation of CBP20 activity in nuclear lysates (see Fig. 4*B*)). Creatine phosphate, ATP, and m^7GpppG-capped precursor adenovirus mRNA were added to initiate splicing (see "Experimental Procedures"). Extracts prepared from quiescent cells were not competent to splice the m^7GpppG-capped precursor RNA (Fig. 6). However, splicing of the m^7GpppG-capped RNA was markedly stimulated in extracts prepared from heregulin-treated cells and was ~5-fold higher than the splicing of a nonspecific ApppG-capped RNA probe by the same extract (data not shown). These results indicate that under conditions where growth factor signaling activates CBC, there is a corresponding stimulation in capped precursor mRNA splicing. Because we also observed some increase in ApppG-capped RNA splicing, the possibility exists that other targets, perhaps acting in conjunction with the CBC, may be important in mediating the observed growth factor effect in cap-dependent RNA splicing. Thus, cap-dependent RNA splicing, in addition to CBC-capped RNA binding, is a functional end point for growth factor-coupled signaling pathways leading to the nucleus.

DISCUSSION

The original goal of these studies was to identify novel nuclear activities that were susceptible to growth factor regulation to further our understanding of how growth factors exert their effects in the nucleus. Using a photoaffinity labeling approach to detect nuclear proteins that specifically incorporate [α-^{32}P]GTP, we detected an 18-kDa nuclear activity that was highly sensitive to the addition of growth factors to G_0 phase-arrested cells. The fundamental role of this activity in cell growth regulation is underscored by its response to growth factors, its specific association with the G_1/S phase of the cell cycle, its activation under conditions of cell stress, and the fact that we have found this activity in every cell and tissue type examined thus far. It was therefore interesting to find that the 18-kDa activity corresponds to the RNA cap-binding protein CBP20, suggesting a necessity for a regulated nuclear cap binding event in cell growth control.

The m^7G(5′)ppp(5′)N cap structure on RNAs transcribed by RNA polymerase II has been known for some time to be important for the stability of these RNAs (25, 26) and to facilitate different aspects of RNA metabolism, including translation initiation, pre-mRNA splicing, and nuclear transport. In recent years, CBP20 and its 80-kDa binding partner, CBP80 (collectively termed CBC), have been identified as the protein complex that binds to the cap structure in the nucleus and mediates the cap-dependent enhancement of pre-mRNA splicing and export of U snRNAs (7, 9). To our knowledge, this is the

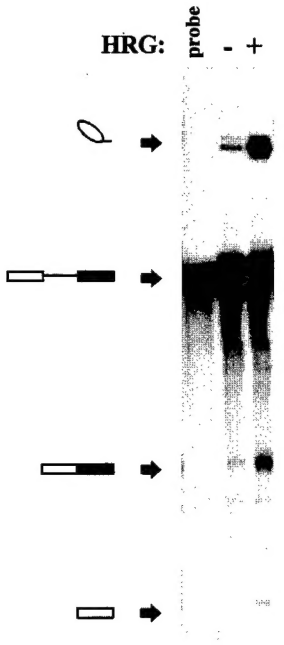


FIG. 6. **Splicing of m^7GpppN-capped RNAs is initiated by growth factor receptor-coupled signal transduction to the nucleus.** Splicing extracts were prepared from HeLa cells that were either serum-starved or starved and then stimulated with heregulin (*HRG*) (100 nN) for 24 h. These lysates were then assayed for their ability to support splicing of an m^7GpppG-capped pre-mRNA probe. The mature splice products and intermediates of the splicing reaction are indicated diagrammatically on the left. The results shown are representative of three experiments.

first report describing a regulated binding activity by the CBC and thus implies that RNA metabolic processes ascribed to the CBC will be regulated as well. This is supported by our finding that growth factors regulate the *in vitro* splicing of precursor mRNA in nuclear lysates from HeLa cells.

An understanding of the signaling processes that lead to CBC activation could shed light on how mitogens influence gene expression by modulating RNA metabolism. All indications are that the CBC may receive inputs from multiple pathways. The Ras-Raf-MEK-extracellular receptor-activated kinase signaling cascade is one pathway that is central to mediating growth factor effects in the nucleus, and we have observed that expression of oncogenic Ras G12V in cells results in an activation of the CBC. Stress-activated signaling pathways also induce CBC activation. There are a number of lines of evidence that indicate that signaling pathways stimulated by Rho-like GTP-binding proteins (*e.g.* Cdc42 and Rac) both participate in cellular stress responses (4–7, 27) and are under growth factor control (25–27). In fact, we have found that activated forms of Cdc42 give rise to an effective activation of the CBC. Given that Cdc42 has been suggested to input into rapamycin-sensitive pathways involving FRAP (FKB12/rapamycin-associated protein) by activating the p70 S6 kinase (27), it is interesting to consider whether the regulation of the CBC is linked to translational control. The cytosolic mRNA cap-binding protein eIF-4E, which plays a critical role in a number of mRNA translational events (29), is also susceptible to growth factor regulation. The phosphorylation of eIF-4E occurs in response to multiple growth factors (including NGF in PC12 cells) and cell cycle arrest (28, 30) and appears to occur downstream of multiple signaling pathways, including the extracellular receptor-activated kinase, c-Jun N-terminal kinase/stress-activated protein kinase, and p38 kinase pathways (29). In addition to its direct phosphorylation, the activity of eIF-4E is also regulated by two other growth factor-responsive factors, the eIF-4E-binding proteins 4E-BP1 and 4E-BP2 (30), and recently, 4E-BP1 has shown to be phosphorylated by the phosphatidylinositol 3-kinase-related kinase FRAP (31). Thus, it will be interesting to see if the cytosolic cap-binding protein eIF-4E and the CBC are similarly or even coordinately regulated through growth factor-initiated signals.

A growth factor-dependent phosphorylation of CBP20 could have a direct effect on its RNA cap-binding activity (similar to eIF-4E), although thus far, we have not been able to detect a growth factor-stimulated phosphorylation of CBP20 *in vivo*. The cellular levels of CBP20, its ability to bind CBP80, and its nuclear localization are not affected by growth factor stimulation (see Fig. 2). We are currently examining whether growth factors influence the interactions between the CBC and specific regulatory proteins to stimulate the binding of the CBC to capped RNA in a manner analogous to the growth factor-regulated interaction between eIF-4E and the 4E-BP proteins.

Our demonstration that the CBC is susceptible to extracellular regulation, in conjunction with the previously defined role for the CBC in RNA processing, makes the CBC an attractive candidate for translating growth factor signals into altered gene expression by affecting the metabolism of specific subsets of RNAs. However, given that the CBC affects both the processing and transport of RNAs transcribed by RNA polymerase II, the growth factor-dependent binding of the CBC to capped RNA may result in a general regulation of gene expression. The reduced ability of the CBC to bind capped RNAs in the absence of a growth factor signal could serve as a checkpoint for cell growth by guarding against the further processing of inappropriate or "leaky" transcripts. This suggests that altered levels and/or mutations of the CBC might be capable of deregulating cell growth. Future studies will be directed toward determining how growth factors influence different aspects of RNA processing (including precursor mRNA splicing and RNA export) through the CBC and how overexpression and/or mutation of the CBC impacts upon normal cell growth.

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